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*Published in:*
The Journal of Biological Chemistry

*DOI:*
10.1074/jbc.274.48.34219

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*Document Version*
Publisher's PDF, also known as Version of record

*Publication date:*
1999

Link to publication in University of Groningen/UMCG research database

*Citation for published version (APA):*
Knoblauch, N. T. M., Rudiger, S., Schonfeld, H. J., Driessen, A. J. M., Schneider-Mergener, J., & Bukau, B. (1999). Substrate specificity of the SecB chaperone. *The Journal of Biological Chemistry*, *274*(48), 34219 - 34225. https://doi.org/10.1074/jbc.274.48.34219

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 48, Issue of November 26, pp. 34219–34225, 1999
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*Printed in U.S.A.*

# Substrate Specificity of the SecB Chaperone*

(Received for publication, July 20, 1999, and in revised form, September 9, 1999)

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**The bacterial chaperone SecB assists translocation of proteins across the inner membrane. The mechanism by which it differentiates between secretory and cytosolic proteins is poorly understood. To identify its binding motif, we screened 2688 peptides covering sequences of 23 proteins for SecB binding. The motif is ~9 residues long and is enriched in aromatic and basic residues, whereas acidic residues are disfavored. Its identification allows the prediction of binding regions within protein sequences with up to 87% accuracy. SecB-binding regions occur statistically every 20–30 residues. The occurrence and affinity of binding regions are similar in SecB-dependent and -independent secretory proteins and in cytosolic proteins, and SecB lacks specificity toward signal sequences. SecB cannot thus differentiate between secretory and non-secretory proteins via its binding specificity. This conclusion is supported by the finding that SecB binds denatured luciferase, thereby allowing subsequent refolding by the DnaK system. SecB may rather be a general chaperone whose involvement in translocation is mediated by interactions of SecB and signal sequences of SecB-bound preproteins with the translocation apparatus.**

SecB is a bacterial chaperone that assists translocation of precursor proteins across the cytoplasmic membrane (1–4). It associates with newly synthesized precursors, either late during or shortly after translation, and thereby maintains them in a translocation-competent state (5–10). SecB of *Escherichia coli* is furthermore known to interact with the SecA subunit of the translocase (7, 11), a feature that contributes to the apparent dedication of SecB to assist protein translocation.

Genetic analysis of *secB::Tn5* mutant cells identified periplasmic and outer membrane proteins of *E. coli* proteins that are translocated dependently (*e.g.* maltose-binding protein (MBP),[1] galactose-binding protein (GBP), oligopeptide-binding protein (OppA), LamB, OmpA, and OmpF) or independently (*e.g.* ribose-binding protein, alkaline phosphatase, β-lactamase, and outer membrane lipoprotein) of SecB (6, 12–14). SecB-independent secretory proteins may utilize other cytosolic chaperones for translocation, including GroEL (15–17) and the DnaK system (14, 18), but may possibly utilize SecB as well.

SecB is a homotetramer composed of 17-kDa subunits (19–22) and whose interaction with substrates is independent of ATP (1, 23). The principles governing substrate selection by SecB are controversial. Based on their finding that, *in vitro*, SecB has 100-fold higher affinity for denatured signal sequence containing MBP precursor than for the corresponding mature protein, Watanabe and Blobel (19, 24) proposed that SecB recognizes the signal sequence directly. On the other hand, signal sequences were dispensable for association of SecB with other substrates, including MBP, both *in vitro* (25, 26) and *in vivo* (6, 27), although they might contribute to SecB binding, *e.g.* in the case of LamB (28). Randall and co-workers (1, 23, 29) postulated a kinetic partitioning model, according to which SecB does not bind specifically to the signal sequence, but to various segments of the precursor polypeptide. The role of the signal sequence in this model is to reduce the folding rate of newly synthesized precursors to allow association with SecB. The refolding rate of unfolded MBP is indeed faster in the absence than in the presence of the signal sequence (30, 31), and SecB is unable to associate with refolding mature MBP unless a mutation in mature MBP is introduced that slows down the folding rate without affecting the thermostability of the folded protein. This kinetic partitioning model has been challenged by stopped-flow kinetic measurements of SecB interactions with denatured bovine pancreatic trypsin inhibitor (BPTI) and barnase (32, 33). Association of SecB with these substrates is nearly diffusion-limited and therefore orders of magnitude faster than folding of newly synthesized polypeptides with or without signal sequences. Since the folding rates of newly synthesized proteins are in any case slower than the SecB association rate, they cannot therefore account for the ability of SecB to discriminate between cytosolic and exported proteins as required for the kinetic partitioning model. Recognition of high affinity binding sites in the precursor polypeptide might thus be a more important determinant for SecB substrate specificity than the folding rate of the substrate (32).

The sequence motif that is recognized by SecB is poorly understood. In the case of MBP (34), GBP (35), and OppA (36), SecB binds to multiple fragments covering a large fraction (~50%) of the primary sequence. The SecB tetramer has a binding site for positively charged peptides, the occupation of which is proposed to result in conformational changes that expose hydrophobic binding sites (37).

We determined the substrate specificity of SecB by screening cellulose membrane-coupled peptide scans of protein sequences for SecB binding as well as the potential of SecB to assist the

* This work was supported by grants from the Deutsche Forschungsgemeinschaft, Bundesministerium für Forschung und Technologie, and the Fonds der Chemischen Industrie (to B. B. and J. S.-M.) and by a PIONIER grant from the Netherlands Organization for Scientific Research (to A. J. M. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MBP, maltose-binding protein; GBP, galactose-binding protein.



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folding of non-secretory proteins. These approaches allowed us to elucidate principles of action of this chaperone.

## EXPERIMENTAL PROCEDURES

*Purification of SecB*—The *secB* gene was amplified by polymerase chain reaction using chromosomal DNA as template and cloned downstream of the isopropyl-β-D-thiogalactopyranoside-regulatable promoter of the pREP4 expression plasmid. Overexpression of *secB* was induced by addition of isopropyl-β-D-thiogalactopyranoside to the cell culture grown in double-concentrated Luria broth medium. About 30 g of wet bacterial cells obtained from 5 liters of fermentation broth was resuspended on ice in 140 ml of 50 mM Tris-HCl (pH 7.4) containing 10 mM benzamidine hydrochloride, 5 mM ϵ-aminocaproic acid; 5 mM EGTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride; 0.1 mM *o*-phenanthroline, 0.1 mM 3,4-dichloroisocoumarin, 50 μM E-64, 10 μM leupeptin, and 100 units/ml aprotinin. After supplementation with 1.4 mg of DNase (10 μg/ml) and 14 mg of lysozyme (100 μg/ml), cells were sonified for 10 min (Branson sonifier, medium rod, 50% cycle, 20 watts). After opening of cells by sonication, insoluble material was removed by centrifugation. SecB was precipitated from the supernatant by addition of ammonium sulfate up to 35% relative saturation, resolubilized in 50 mM piperazine-HCl (pH 5.0) (piperazine buffer), and then extensively dialyzed against the same buffer. After filtration through a 0.45-μm pore-sized filter, the solution was applied to a Q-Sepharose Fast Flow column (2.6 × 15 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with piperazine buffer. After washing, bound material was eluted by a salt gradient. Fractions containing SecB as analyzed by SDS-polyacrylamide gel electrophoresis were pooled; adjusted to pH 7.4; and then applied to a Sephacryl S-300 HR column (2.6 × 100 cm) equilibrated with 10 mM sodium phosphate (pH 7.4) and 140 mM NaCl (~100 mg of SecB load/run). Fractions containing purified SecB were pooled and stored frozen at −80 °C. About 100 mg of homogeneous SecB was obtained from 1 liter of fermentation broth. Ion spray mass spectroscopy, N-terminal sequencing, and amino acid analysis verified the authenticity of the purified protein. Recombinant SecB started with serine at the amino terminus and was not blocked, in contradiction to what was reported earlier (20, 38). Purified SecB was protected against proteinase K cleavage by reduced and *S*-carboxyamidomethylated bovine pancreatic trypsin inhibitor (BPTI) or by the peptides melittin, mastoparan, or bradykinin as described (37). At concentrations below 0.7 g/liter, SecB formed an equilibrium between tetramers and monomers as determined by analytical ultracentrifugation (21). The SecB mutants L75Q and E77K were purified as described previously (39).

*Luciferase Aggregation and Refolding Assays*—A stock solution of firefly luciferase (Sigma; 64 μM in 1 M glycylglycine (pH 7.4)) was diluted 6.4-fold into unfolding buffer (25 mM HEPES/KOH (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 6 M guanidinium chloride) and denatured by incubation at room temperature for 5 min. Denatured luciferase was diluted to an 80 nM final concentration into refolding buffer (25 mM HEPES/KOH (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 5 mM ATP) containing 800 nM DnaK, 160 nM DnaJ, and 200 nM $(GrpE)_2$ where indicated. Aliquots of 1 μl were diluted into 125 μl of assay buffer (25 mM glycylglycine (pH 7.4), 5 mM $MgSO_4$, and 5 mM ATP) and analyzed for bioluminescence activity in a Biolumat (Berthold) as described previously (40).

*Screening of Cellulose-bound Peptides for SecB Affinity*—Peptide libraries were prepared by automated spot synthesis (41–43) using the software LISA (Jerini BioTool GmbH, Berlin, Germany). Peptides were C-terminally attached to cellulose via a $(\beta\text{-Ala})_2$ spacer. Peptides were derived from protein sequences of *E. coli* (GBP, LamB, MBP, OmpA, OmpF, alkaline phosphatase, β-lactamase, outer membrane lipoprotein, ribose-binding protein, FtsZ, SecA, $\sigma^{32}$, and ribosomal protein L2), *Saccharomyces cerevisiae* (cytochrome $b_2$, ATP synthase β-chain (F1β), prepro-α-factor, and ATP synthase protein 9 (Su9)), pig (citrate synthase), cattle (bovine pancreatic trypsin inhibitor), mouse (dihydrofolate reductase), *Photinus pyralis* (luciferase), and bacteriophage λ (λCI and λO). Before screening, the dry membranes were washed for 10 min in methanol and 3 × 20 min in Tris-buffered saline (31 mM Tris-HCl (pH 7.6), 170 mM NaCl, and 6.4 mM KCl). SecB (200 nM) was allowed to react with peptide scans in MP2 buffer (31 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, 0.05% Tween 20, and 5% sucrose) for 40 min at 25 °C with gentle shaking. Unbound SecB was removed with Tris-buffered saline (4 °C), and peptide-bound SecB was electrotransferred onto polyvinylidene difluoride membranes (Millipore Corp.) as described (44). Polyvinylidene difluoride membranes were sandwiched between blotting papers soaked with XK buffer (75 mM Tris base, 120 mM 6-aminohexanoic acid, and 0.01% SDS) and the anode buffers XA1 (90 mM Tris base) and XA2 (300 mM Tris base) kept at 4 °C. Transferred SecB was detected by SecB-specific polyclonal rabbit sera, an alkaline phosphatase-conjugated secondary antibody, and enhanced chemifluorescence measurement (ECF kit, Amersham Pharmacia Biotech) using a fluoroimaging system (FLA2000, Fuji). Quantification was performed using TINA 2.10g (Raytest, Straubenhardt, Germany), and the relative intensities have been normalized to the averaged signal of the reference peptides AKTLILSHLRFVV, VVHIARNYAGYG, and QRKLFFNLRKTKQ, which was set as 100. Peptides were grouped into four SecB-binding classes relative to this signal (class 1, high affinity, relative SecB affinity value of ≥43.5; class 2, medium affinity, <43.5 and ≥16.5; class 3, low affinity, <16.5 and ≥4.5; and class 4, no affinity, <4.5).

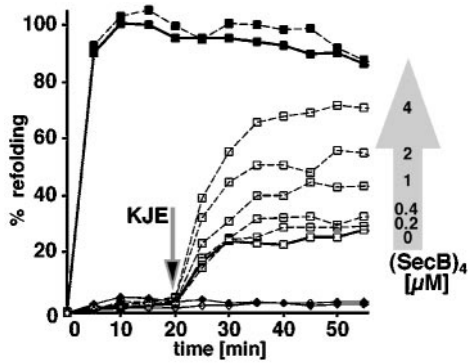


FIG. 1. **Transfer of unfolded luciferase from SecB to the DnaK system.** Guanidinium-unfolded luciferase was diluted into refolding buffer (80 nM final concentration) and tested for activity. ♦, no chaperones added; ◇, bovine serum albumin (5.5 μM); ■——■, DnaK, DnaJ, and GrpE; ■– – –■, DnaK, DnaJ, GrpE, and $(SecB)_4$ (2 μM); □——□, DnaK, DnaJ, and GrpE (KJE; after 20 min); □– – –□, $(Sec B)_4$ (0.2–4 μM; at time 0) and KJE (after 20 min). The DnaK system was added in the following concentrations: 800 nM DnaK, 160 nM DnaJ, and 200 nM $(GrpE)_2$. The maximal refolding yield obtained with DnaK, DnaJ, and GrpE was set as 100%, which corresponds to ~80% of the native control. The inability of SecB to refold luciferase in the absence of the DnaK system is shown in the order-of-addition experiments (□).

## RESULTS

*SecB Can Cooperate with the DnaK System in Protein Refolding in Vitro*—To elucidate the substrate specificity of SecB, we investigated the degree of specialization of SecB for secretory proteins. We tested *in vitro* whether SecB has the capability to assist folding of non-secretory proteins using firefly luciferase as substrate. Luciferase is cytosolic when produced in *E. coli* and requires the activity of the DnaK chaperone system for efficient refolding after thermal or chemical denaturation (40, 45–47).

Guanidinium-denatured luciferase did not refold spontaneously or in the presence of bovine serum albumin (Fig. 1). SecB, even when added at a 20-fold molar excess of the SecB tetramer over luciferase, did not affect the rate of spontaneous refolding. In contrast, when DnaK and its co-chaperones DnaJ and GrpE (at a 10:2:5-fold molar excess over luciferase) were present in refolding buffer at time 0 when luciferase was added, luciferase refolded with a high rate to ~80% of the native control. The simultaneous presence of SecB at an even higher concentration (25-fold molar excess) did not affect the rate and yield of luciferase refolding, indicating that SecB does not interfere with the chaperone activity of the DnaK system. However, at this high concentration (25-fold molar excess over luciferase), SecB was capable of preventing aggregation of denatured luciferase for at least 1 h as measured by light scattering (data not shown). Although it is a high concentration, it is still below the physiological concentration of SecB (~13 μM tetramer).[2] To test whether SecB can cooperate with the DnaK system in luciferase refolding, we performed order-of-addition experiments (Fig.

[2] E. Schaffitzel, unpublished data.

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1) in which the DnaK system was added 20 min after addition of luciferase to the refolding mixture. In the absence of SecB, this late addition reduced the refolding yield to ~25% of the maximal value due to aggregation of luciferase. Increasing concentrations of SecB present at time 0 steadily increased the rate and yield of luciferase refolding by the DnaK system (added at 20 min), with the highest refolding yield obtained at a 50-fold molar excess of SecB tetramer over luciferase (Fig. 1). The refolding yields were higher when DnaK was added already at 10 min after addition of luciferase (data not shown), indicating that SecB holds luciferase in a folding-competent state for only a limited time. These experiments indicate that SecB can act as a "holder" chaperone that prevents aggregation and that cooperates with DnaK in refolding of a non-native cytosolic protein.

*Screening of Peptide Scans for Binding to SecB*—To determine the binding motif within protein sequences recognized by SecB, we screened cellulose-bound peptide scans (48) representing the complete sequences of 23 proteins for SecB binding. The peptide scans were composed of 13-mer peptides that overlap by 10 residues and therefore present all potential linear binding sites for SecB. A subset contains secretory proteins represented in the library as precursors with signal sequences. They are either prokaryotic proteins translocated in a SecB-dependent (GBP, LamB, MBP, OmpA, and OmpF) or -independent (alkaline phosphatase, $\beta$-lactamase, outer membrane lipoprotein, and ribose-binding protein) manner or eukaryotic proteins translocated into the endoplasmic reticulum (bovine pancreatic trypsin inhibitor (BPTI) and prepro-$\alpha$-factor) or mitochondria (citrate synthase, cytochrome $b_2$, ATP synthase $\beta$-chain (F1$\beta$), and ATP synthase protein 9 (Su9)). For comparison, a subset of non-secretory proteins was screened (dihydrofolate reductase, FtsZ, luciferase, SecA, $\sigma^{32}$, L2, λCI, and λO). They were incubated with SecB to equilibrium, followed by electrotransfer and immunodetection of the chaperone. Fig. 2 shows selected peptide scans.

SecB bound only to a subset of the peptides, indicating that it differentiates between amino acid side chains. It bound frequently to neighboring peptides in the scan, indicating that a SecB-binding site is shared by these peptides. SecB-binding peptides existed in all peptide scans tested, with no apparent clustering within the scans (*e.g.* at N or C termini). Sequence alignment of overlapping binding peptides allowed definition of SecB-binding regions (see below). These occurred frequently, every 20–30 residues within the protein sequences tested. This frequency was not affected by cellular and organellar origin, size, and oligomeric status of the proteins and did not differ between secretory and non-secretory and homologous and heterologous proteins. SecB thus has broad substrate-binding specificity at the peptide level, which by itself cannot differentiate between substrates and non-substrates.

The SecB mutants L75Q and E77K associate with precursor polypeptides, but are deficient in SecA interaction (39). We compared the specificity of these mutants with wild-type SecB for association with 76 peptides derived from the λCI sequence and found identical binding patterns (data not shown). This suggests that the SecB peptide-binding site is different from the SecA recognition site.

*Amino Acid Distribution within SecB-binding Peptides*—The large data set allowed reliable statistical analysis of the substrate motif recognized by SecB. All screened peptides were grouped into four classes according to their affinity for SecB (high, medium, low, and no affinity) as determined by fluoroimager quantification of the SecB signals (Fig. 3*A*).

The relative occurrence of the 20 amino acids in the peptide library is similar to what is found in natural proteins, except for a low representation of Cys (44) (data not shown). Substantial differences existed between the amino acid distribution of SecB-binding and non-binding peptides (Fig. 3*B*). Medium affinity SecB-binding peptides are enriched in basic residues (Arg and Lys). High affinity SecB binders are, in addition, up to 2-fold enriched in aromatic residues (Phe, Tyr, and Trp). Acidic residues (Asp and Glu) are strongly disfavored, and most other residues are slightly disfavored. Large hydrophobic residues (Ile, Leu, and Val) are not enriched.

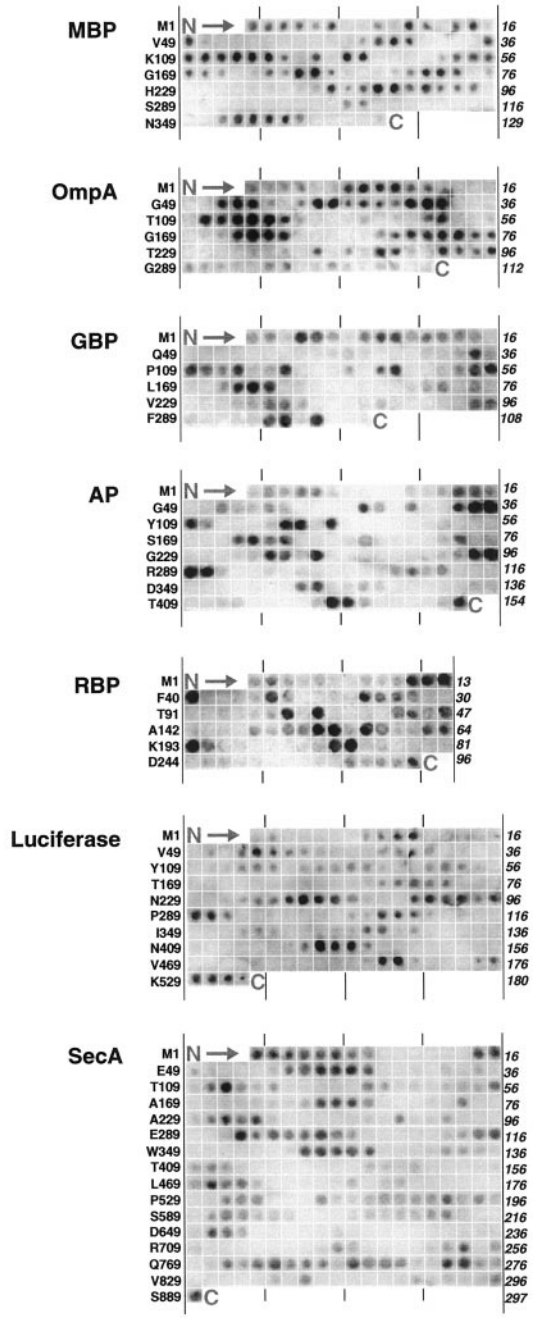


FIG. 2. **SecB binding to cellulose-bound peptide scans.** Peptide scans derived from precursor sequences of the *E. coli* proteins MBP, OmpA, GBP, alkaline phosphatase (*AP*), and ribose-binding protein (*RBP*) and from the sequences of *P. pyralis* luciferase and *E. coli* SecA were screened for SecB binding. The *letters* on the *left* indicate the first residue of the first spot of each row. The *italic numbers* on the *right* indicate the spot number of the last spot of each row.

*Sequence Motif Recognized by SecB*—94 regions with high affinity for SecB were aligned to identify the consensus binding motif. It consists of ~9 neighboring residues, as judged from

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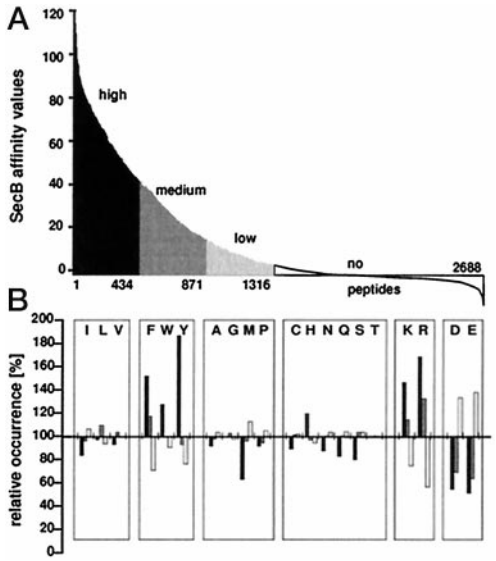


FIG. 3. **Amino acid distribution in peptide scanning libraries.** For 2688 peptides representing 23 protein sequences, the relative amino acid occurrence was determined. *A*, normalized affinity of SecB for the peptides investigated. Peptides are ordered according to their SecB affinity (class 1, *black*; class 2, *dark gray*; class 3, *light gray*; and class 4, *white*). *B*, comparison of peptides of class 1 (high SecB affinity; *black bars*), classes 2 and 3 (medium and low SecB affinity; *gray bars*), and class 4 (no SecB affinity; *white bars*). The numbers for each amino acid are normalized to its occurrence in the whole peptide library (set as 100). The differences between class 1 and class 4 populations for Val, Gly, Cys, and Thr are not statistically significant in the $\chi^2$ test ($p > 0.05$).

the fact that a continuous stretch of that length showed significant changes in amino acid composition compared with the total library. The binding motif is enriched in basic and aromatic residues, whereas acidic residues are disfavored unless localized in the neighborhood of basic residues (Fig. 4). The relative enrichment of aromatic residues in SecB-binding peptides is lower compared with the enrichment of hydrophobic residues in DnaK-binding peptides (44). This is a consequence of the lower abundance in the peptide library of aromatic residues (8.1%) as compared with hydrophobic residues (26%). SecB-binding regions contain each at least 3 aromatic or basic residues. The presence of each acidic residue in a SecB-binding region has to be compensated by another basic residue. Furthermore, in the absence of aromatic residues, at least four net positive charges are required. The positioning of charged and aromatic residues within the motif is not important, except that 2 aromatic residues in the direct neighborhood do not contribute more to SecB affinity as compared with only 1 residue.

We compared the presence of these identified features of the motif within SecB-binding regions with the experimental data of five peptide scans comprising 2209 amino acids (alkaline phosphatase, LamB, luciferase, MBP, and OmpA) (Table I). These features existed in 79% of the 53 high affinity binding regions if the considered consecutive stretch between basic and aromatic residues had a length of up to 9 residues, and only four predicted regions were not high affinity binders (Table I). In the case of a length of up to 10 residues, the prediction of correctly identified regions increased to 87%, with seven incorrect predictions of SecB-binding regions. Together, the identified motif allows a precise description of SecB-binding regions in protein sequences.

*Localization of SecB-binding Regions within Native Protein Structures*—We determined the localization of identified SecB-binding regions within the folded structures of several proteins, including alkaline phosphatase, MBP, OmpA, LamB, and luciferase (see Fig. 5 for MBP and OmpA). SecB did not show binding preference for specific secondary structure elements. Positively charged side chains of SecB-binding regions are exposed in several cases. In the case of the proteins that are not outer membrane proteins, most aromatic side chains that characterize high affinity binding regions are buried (*e.g.* only 6 out of 23 SecB-binding regions within MBP are exposed). The buried nature of these regions explains why SecB is unable to stably interact with the native conformers of these substrates, as shown in particular for MBP (26). Exposure of single side chains within SecB-binding sequences is obviously not sufficient for SecB binding. In contrast, in the case of outer membrane proteins, significantly more high affinity binding regions for SecB are surface-exposed. In particular, rings of aromatic residues that position the transmembrane segments of outer membrane proteins in the lipid bilayer (49) constitute SecB-binding regions, *e.g.* in OmpA (Fig. 5). SecB is known to bind solubilized OmpA *in vitro* (50), which agrees well with our study.

*Affinity of SecB for Signal Sequence Peptides*—It has been postulated that SecB can differentiate between secretory and non-secretory proteins as well as between SecB-dependent and -independent proteins by recognition of the signal sequence (19, 24). To investigate this hypothesis, we studied SecB binding to peptides derived from signal sequences (Fig. 6). Within the SecB-dependent proteins tested, we found that some signal sequences have high affinity for SecB (*e.g.* MBP), whereas others have only low affinity for SecB (*e.g.* OmpF). Similarly, among the SecB-independent proteins tested, some signal sequences have affinity for SecB (*e.g.* alkaline phosphatase), whereas others have low affinity (*e.g.* outer membrane lipoprotein). Furthermore, the distribution of SecB-binding and non-binding peptides is statistically the same within both signal sequence peptides and the whole peptide library as well as between secretory and non-secretory proteins. We nevertheless observed that SecB has much higher affinity for signal sequences of mitochondrial precursors than for those of *E. coli* precursor proteins (data not shown). Together, these findings rule out the possibility that differences in the recognition of signal sequences are the basis for substrate selection by SecB.

## DISCUSSION

We determined the principles governing substrate recognition by the SecB chaperone employing cellulose-bound peptide scans. This approach avoids solubility problems of hydrophobic peptides and allows screening of thousands of peptides, which permits identification of the binding motif and all potential linear binding sites within the tested protein sequences. It was successfully established to dissect the substrate specificity of the *E. coli* DnaK chaperone (44, 51). This peptide-based approach is appropriate for SecB since peptides bind SecB with high affinity and compete with protein substrates for binding (37). Peptide studies were in fact the basis for establishment of the kinetic partitioning model for SecB substrate selection (1).

We were concerned about the possibility that the binding of SecB to peptides does not reflect SecB-substrate interactions, but instead the high affinity interaction of SecB with the C terminus of SecA. The C terminus of SecA indeed contains a SecB-binding region, which, however, is not among the strongest SecB-binding regions identified in our screen. Furthermore, SecB mutants that have lost the ability to interact with SecA showed the same substrate-binding pattern as wild-type SecB. In addition, recent evidence demonstrates that the C terminus of SecA coordinates a zinc ion that is needed for high affinity binding of SecB (52), and zinc was not present in our experiments. These findings indicate that the observed SecB-binding

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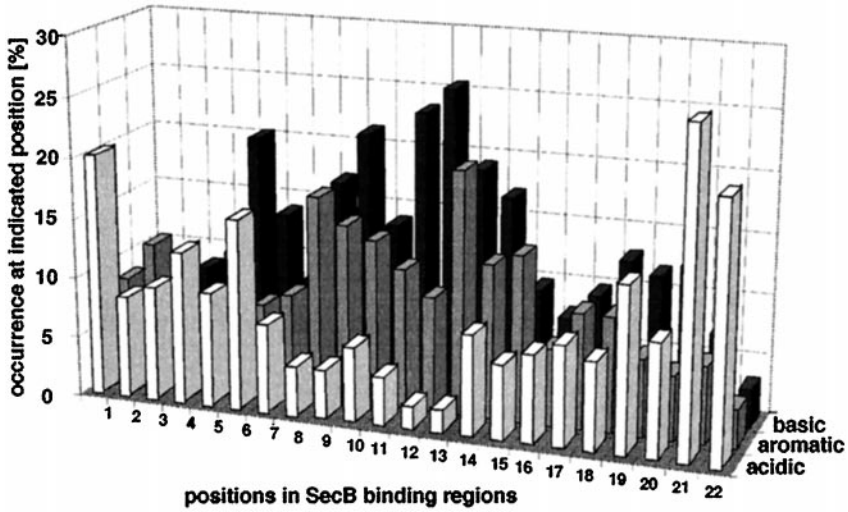


FIG. 4. **Sequence alignment of SecB-binding regions.** 94 SecB-binding regions each constituting a single strong SecB-binding region were aligned. The frequency of acidic (Asp and Glu; *white bars*), aromatic (Phe, Trp, and Tyr; *gray bars*), and basic (Arg and Lys; *black bars*) residues at each position is given as a percentage.

TABLE I
*Prediction of high affinity SecB-binding regions in five protein sequences*

High affinity SecB-binding regions were predicted as consecutive peptide sequences of 9 or 10 residues according to the following rules. (i) They contain at least 3 aromatic or basic residues. (ii) The presence of each acidic residue in a SecB-binding region has to be compensated by another basic residue. (iii) In the absence of aromatic residues, at least four net positive charges are required. (iv) The positioning of charged and aromatic residues within the motif is not important, except that 2 aromatic residues in the direct neighbourhood do not contribute more to SecB affinity as compared with only 1 residue. Experimentally determined SecB regions were taken from peptide scans of alkaline phosphatase, LamB, luciferase, MBP, and OmpA.

| | Maximum length of predicted regions | |
|---|---|---|
| | 9 residues | 10 residues |
| Total regions | | |
| Experimentally determined | 53 (100%)[a] | 53 (100%) |
| Predicted | 46 | 53 |
| Correct predicted regions | 42 (79%) | 46 (87%) |
| False predicted regions | 4 (8%) | 7 (13%) |

[a] Values in parentheses represent percentage of the total number of experimentally determined high affinity SecB-binding regions.

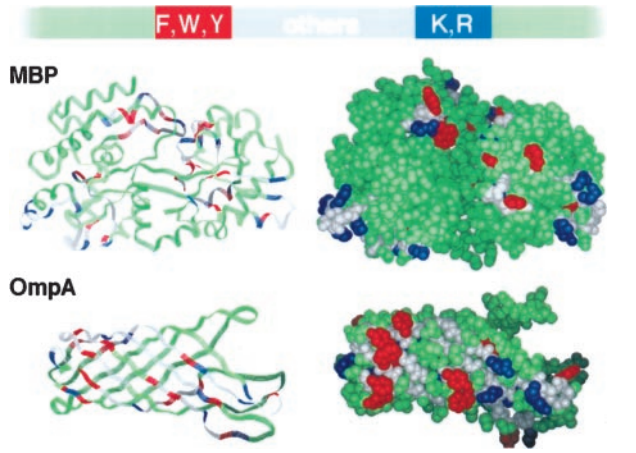


FIG. 5. **Localization of SecB-binding regions in native protein structures.** Ribbon and space-filling representations (Insight II, MSI, Inc.) of the structures of the corresponding native proteins of mature MBP (56) and the transmembrane segment of OmpA (49) are shown. *Red* and *blue* side chains indicate aromatic and basic residues, respectively, of regions with high affinity for SecB identified within the peptide scans (see Fig. 2). The backbones of these segments are *gray*.

signals in our screen reflect true interactions of SecB with substrates via its substrate-binding site.

We considered that the binding of SecB to positively charged residues is unspecific, given that SecB is an acidic protein (20). If this were the case, one would expect two populations of SecB-binding peptides, positively charged peptides and peptides enriched in aromatic residues. There is, however, only one population of SecB-binding peptides since the number of positively charged residues is connected to the number of aromatic residues. Thus, peptides with 2 aromatic residues bind SecB only if there is an additional Arg or Lys, whereas peptides with 3 aromatic residues bind in the absence of Arg or Lys. In all cases, SecB binding can be inhibited by the presence of acidic residues within the peptides, providing a further indication that the recognition of basic and aromatic residues is a linked event. We can also rule out the possibility that recognition of aromatic residues is an unspecific hydrophobic interaction since Leu, Ile, and Val are not enriched in the binding peptides.

The characteristics of the SecB-binding regions identified in this study are in agreement with and extend earlier findings of studies investigating the affinity of SecB for fragments of protein substrates and selected peptides (34–37). Furthermore, our approach allows, for the first time, the identification of the SecB-binding motif and the prediction of SecB-binding regions within protein substrates. The motif consists of a continuous stretch of ~9 residues enriched in basic and aromatic residues, whereas acidic residues are strongly disfavored. In contrast, large hydrophobic aliphatic residues are not enriched. This indicates that SecB has binding pockets or surfaces that are specific for aromatic residues. The aromatic side chains of high affinity SecB-binding regions typically occur within core regions of folded proteins, as shown for MBP (Fig. 5). The nature of this substrate-binding motif allows SecB to bind preferentially to unfolded conformers of protein substrates and thus forms a basis for its function as a chaperone.

The SecB-binding motif shares overall similarity with the motif recognized by the DnaK chaperone in that both motifs comprise a hydrophobic patch in which negatively charged residues are disfavored (44, 53). Differences exist with respect to the length of this patch (~9 residues for SecB and 4–5 residues for DnaK), the positioning of basic residues (within this patch for SecB and outside for DnaK), and the nature of hydrophobic residues. Whereas SecB favors aromatic residues, DnaK favors large hydrophobic residues with a strong preference for leucine (44). Despite these differences, many binding sites for SecB and DnaK are shared. Thus, the luciferase sequence contains 13 high affinity binding sites for DnaK and nine for SecB, eight of which are common to both (if adjacent

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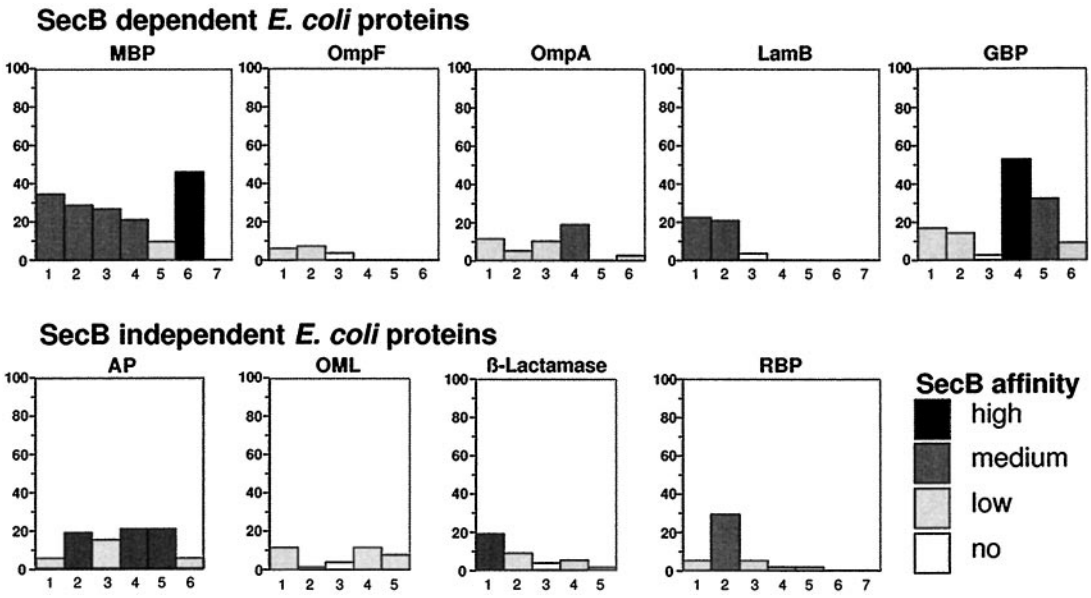


FIG. 6. **SecB association with signal sequences.** The SecB affinity of 13-mer peptides as determined by scanning the signal sequences of secretory *E. coli* proteins is indicated on the *ordinate*. The SecB affinity class of each peptide is indicated. The *abscissa* indicates the number of each peptide. The most C-terminal peptide with the highest number still contains at least 6 residues of the signal sequence; the three C-terminal peptides also therefore contain N-terminal residues of the mature protein. SecB affinity for signal sequence-derived peptides does not follow the classification into SecB-dependent and -independent proteins.

sites melted to a broad site, they were counted only once). SecB and DnaK therefore have the potential to interact with similar sets of proteins, although additional parameters will clearly also be of relevance, *e.g.* the association rates of SecB-substrate complexes and the kinetics of substrate folding. Similarities in the protein substrate spectra of SecB and DnaK have been established *in vivo* by demonstrating that the DnaK chaperone system can support export of SecB-dependent substrates in *secB* mutant cells (14). Furthermore, we show here that SecB shares with DnaK (40, 46) the ability to prevent aggregation of unfolded firefly luciferase.

Our identification of SecB-binding regions within protein sequences excludes that SecB is able to distinguish between secretory and non-secretory proteins on the basis of differences in binding sites. Of particular importance in this respect is the finding that there is no correlation between SecB dependence of transport and the ability of SecB to bind to signal sequences. Furthermore, the signal sequences of some SecB-dependent secretory proteins do not provide SecB-binding sites of detectable affinity. Our results support a model according to which SecB associates with several internal segments of the mature parts of precursor proteins (54). They also suggest that SecB does not act very early co-translationally since the signal sequences are not prime targets. Our data are instead consistent with the finding for MBP that SecB binds late co-translationally to the nascent polypeptide chain after it has reached a length of ~150 residues (55). An MBP fragment of this length contains six high affinity SecB-binding regions. Furthermore, the MBP segment comprising residues 151–186 that was identified to be required for SecB binding to MBP (6) contains a high affinity SecB-binding region, as found in this study.

On the basis of our findings and the demonstrated fast kinetics of SecB association with substrates (32, 33), it is conceivable that SecB can interact with a large variety of folding proteins even when they are cytosolic proteins. Unfolded firefly luciferase was one such substrate, although a large molar excess of SecB was needed to prevent its aggregation (Fig. 1). This suggests that SecB is capable of binding to a large variety of substrates. Since SecB is the most abundant cytosolic chaperone besides DnaK, with a cellular concentration of SecB (~13 $\mu$M tetramer, half the concentration of DnaK)[2] that is even above that used for the luciferase refolding experiment shown in Fig. 1, the high amount of SecB in the cell might also allow productive interaction with unfolded cytosolic polypeptides. It is unclear to what extent the cell takes advantage of this general chaperone function of SecB to prevent protein aggregation. A mechanistically important finding of our study is that the protein substrate, unfolded firefly luciferase, can dissociate rapidly from association with SecB and be transferred in non-native conformation to the DnaK chaperone system. This rapid dynamic equilibrium between bound and free states may allow the ligand to kinetically partition between folding (1) and rebinding to SecB or, only in the case of secretory proteins, to be transferred to the translocon. The dedicated role of SecB in protein translocation may thus result from events downstream of the SecB-substrate interaction, in particular the association of SecA with SecB and the interaction of the signal sequence with components of the translocon.

*Acknowledgments*—We thank P. Fekkes for the gift of SecB mutant proteins; E. Schaffitzel for unpublished results on SecB levels in *E. coli*; M. Stieger for overexpression of SecB; B. Wipf for fermentation; B. Pöschl for technical assistance; and M. Müller, D. Dougan, and E. Deuerling for reviewing the manuscript.

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